Journal of Medical Virology 54:186–191 (1998)

Human Papillomavirus DNA Sequences and p53 Over-Expression in Laryngeal Squamous Cell Carcinomas in Northeast China

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One-hundred-two patients with laryngeal squamous cell carcinomas in Northeast China were examined for human papillomavirus (HPV) DNA by the polymerase chain reaction (PCR) coupled with Southern blot hybridization, and for p53 over-expression by immunohistochemical staining. HPV DNAs were found in 60 cases (58.8%). HPV-16, -18, -6, -11, and -33 DNAs were detected in 30 cases, 22 cases, 25 cases, two cases, and one case, respectively. In addition, coinfection either with HPV-6 and -16 or with HPV-6 and -18 was detected in 20 cases (33.3% of HPV DNA-positive cases). p53 over-expression was observed in 60 patients (58.8%). p53 was over-expressed significanty in the poorly-differentiated SCC and in patients with metastasis to lymph nodes ($P < 0.05$, respectively). Both HPV DNA and p53-expression were positive in 35 patients, and negative in 17 patients. Either HPV DNA or p53-expression were positive in 50 patients (25 cases each). Although p53 was detected in 35 (58.3%) of HPV-positive patients, there was no significant correlation between HPV infection and p53 over-expression in laryngeal squamous cell carcinomas of Northeast China. ***J. Med. Virol. 54:186–191, 1998.***

KEY WORDS: HPVs; coinfection; p53 expression

INTRODUCTION

Laryngeal squamous cell carcinoma (SCC) is the most frequent malignant tumor in the head and neck and is found predominantly in males. Specifically, the occurence of this carcinoma in females is usually less than 10% of that in males. The incidence of laryngeal SCC in Northeast China is more than four times higher than that of Japan, being approximately 12 per 100,000 population. In addition, approximately 40% of the patients in Northeast China are females, in which almost 80% of carcinomas occur at the supraglottic site.

HPVs have been implicated in proliferative and invasive lesions of the larynx. The low-risk HPV-6 and -11 DNA sequences were detected in approximately 100% of laryngeal papillomatosis [Abramson et al., 1987]. Most of the malignantly transformed papillomas (carcinoma ex-papilloma) were also associated with HPV-6 and -11 [Kashima et al., 1988; Zarod et al., 1988]. The high-risk HPV-16 and/or -18 DNA sequences has been found in high percentage of laryngeal verrucous carcinoma [Brandsma et al., 1986; Perez-Ayala et al., 1990; Fliss et al., 1994]. They were also detected in laryngeal "de novo" SCC as well as in cervical malignancies, while the prevalence of HPV infection in the former has been reported to be much lower than that in the latter [Hosikawa et al., 1990; Perez-Alaya et al., 1990; Anwar et al., 1993].

The high-risk HPV-16, -18, -31, and -33 have the ability to immortalize or transform human fibroblasts and keratinocytes [Pirisi et al., 1993] as well as rodent cell lines in vitro [Tsunokawa et al., 1986; Yasumoto et al., 1986; Kanda et al., 1988]. It has been demonstrated that oncoproteins of E6 and E7 genes of the high-risk HPVs can form complexes with tumor suppressor-gene products of p53 and pRb, respectively [Dyson et al., 1989; Heck et al., 1992]. The interaction of E6 protein with p53 protein promotes degradation of the latter, resulting in the abolishing its tumor suppressor function [Scheffner et al., 1990].

Contract grant sponsor: International Scientific Research Program from the Ministry of Education, Science and Culture of Japan; Contract grant number: Special Cancer Research; 7042009.

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Accepted 21 October 1997

TABLE I. Characteristics of Laryngeal Carcinomas in Northeast China

Characteristic	Male		Female		Statistical significance
Cases	73		29		
Mean age (range)	60.8 y (40–79)		60.2 y (50–68)		
Site					
supraglottic	43		26		*(P < 0.005)
glottic	28	30	2	3	
subglottic	2		1		
Stage (TNM classification)					
T1	7		1		
T2	24		7		
T3	28		13		
T4	14		7		
N0	49		13		
N1	12		10		
N2a	0		1		
N2b	4	24	2	16	*(P < 0.05)
N2c	6		2		
N3	0		1		
M0	73		29		
Histology (differentiation)					
well	20		7		
moderately	47		20		
poorly	6		2		

*Statistical significance was evaluated via chi-square test.

Although genetic and some environmental factors have been considered in the pathogenesis of laryngeal SCC, the etiology is still obscure. To define some of these factors, the HPV DNA sequences and p53 overexpression were investigated in laryngeal "de novo" SCC in Northeast China which has unique epidemiological features in comparison to those in other regions of the world.

SPECIMENS AND METHODS

Specimens

The 102 specimens used in this study were collected during surgical operation at the Department of Otorhinolaryngology, First Clinical College, China Medical University, Shenyan, People's Republic of China. The specimens have been fixed in 10% buffered-formalin (pH 7.4) and embedded in paraffin. The data collected on each patient included age, sex, primary tumor site, stage (TNM classification), and histological grade of tumor differentiation (well, moderately, or poorly differentiated; Table I).

Preparation of DNA

Formalin-fixed, paraffin-embedded specimens were cut into 10 μm thick, and DNA was prepared following the method of Wright and Manos [1990]. DNA was extracted with phenol:chloroform and precipitated with ethanol. Finally, DNA was dissolved in 10 mM Tris-HCl and 1 mM EDTA buffer (TE buffer, pH 7.8).

Polymerase Chain Reaction (PCR)

Primers and PCR conditions used in this study followed those of Fujinaga et al. [1991]. Briefly, the sense primers, pU-1M and pU-31B, are located in the middle of the open reading frame (ORF) E6 and the antisense primer, pU-2R is located in the middle of ORF E7. The sequences of pU-1M, pU-31B and pU-2R were 5′-TGTCAAAAACCGTTGTGTCC-3′, 5′-TGCTAATTCG-GTGCTACCTG-3′, and 5′-GAGCTGTCGCTTAATT-GCTC-3′, respectively. The pU-1M/pU-2R primer pairs amplified 228 bps DNA fragment from both HPV-6 and -11 DNAs. The pU-31B/pU-2R primer pairs amplified 238 bps, 268 bps, 233 bps, 244 bps, 231 bps, and 244 bps DNA fragments from HPV-16, -18, -31, -33, -52b, and -58 DNAs, respectively [Fujinaga et al., 1991]. The cloned HPV-6 DNA and HPV-18 DNA were used as positive controls, respectively.

Agarose Gel Electrophoresis

The PCR product (10 μl) was subjected to 2% agarose gel electrophoresis and photographed under UV-light. For the typing of HPV, PCR product was extracted with chloroform, followed by ethanol precipitation, and suspended in TE buffer. Then, a quarter of the product was digested with *Ava* II, *Rsa* I, *Bgl* II, or *Acc* I in 10 μl reaction mixture for 2 hours at 37°C [Fujinaga et al., 1991]. The digested products were subjected to electrophoresis as described above. When the amplified DNA fragment was digested with *Rsa* I, it was cleaved into 132 bps and 96 bps in the case of HPV-6, 166 bps and 62 bps in the case of HPV-11, and 119 bps and 114 bps in the case of HPV-33, respectively. *Ava* II digestion of PCR product produced 157 bps and 81 bps fragments in the case of HPV-16, 172 bps and 96 bps fragments in the case of HPV-18, and 136 bps and 108 bps fragments in the case of HPV-33, respectively. If HPV-52b were amplified, *Bgl* II digestion of amplified DNA would produce 176 bps and 55 bps fragments, and if it were HPV-58, *Acc* I digestion would produce 126 bps and 118 bps fragments.

Southern Blot Hybridization

After electrophoresis, the DNA was transferred onto Hybond N+ nylon transfer membrane (Amersham, UK) by capillary blotting using 0.4 N NaOH. The PCR products amplified from cloned HPV-6 DNA and cloned HPV-18 DNA were purified from agarose gel by QIAEX II Extraction Kits (Qiagen GmbH and Qiagen Inc., Hilden, Germany) and used as probes, respectively. For the detection of HPV band, hybridization was carried out using the ECL direct labelling and detection kit (Amersham, UK) according to the instructions of the manufacturer.

Immunohistochemical Staining

Sections (5 μm) were mounted on aminoalkylsilane-coated slides (Dako Corp., Carpinteria, CA). After deparaffinization with xylene and rehydration through an ethanol series, the intrinsic peroxidase in tissue sections were inactivated by treatment for 10 minutes with 0.3% solution of hydrogen peroxide in methanol. Then, the sections were treated with Target Retrieval Solution (DAKO Corp.) in boiling water for 20 minutes.

The sections were reacted at room temperature with mouse monoclonal anti-p53 antibody (Mab D07; Novocastra Lab., UK) which has been shown to react with both wild-type and mutant forms of the p53 protein. The signal was detected by avidin-biotin complex (ABC) method (Vector Laboratories, Inc., Burlingame, CA) according to the instructions of the manufacturer. Staining was considered positive when unequivocal dark brown color was observed in the nuclei of tumor cells.

Statistical Analysis

Statistical significance was analyzed via chi-square test.

RESULTS

Characteristics of Laryngeal SCC in Northeast China

A total of 102 laryngeal SCC were collected at surgery from September 1995 to December 1996 (Table I). The ratio of males to females was 2.52:1; that is, there were 73 males and 29 females with ages ranging from 40- to 79-years-old (mean, 61-years-old). The statistical significance between males and females was observed in the site and the staging of laryngeal SCC in Northeast China. The incidence of the supraglottic SCC in laryngeal SCC was higher in females than that in males ($P < 0.005$), and the metastasis to cervical lymph nodes occurred more often in females ($P < 0.05$). No metastasis to other sites was noted in any patient at surgery.

Detection of HPV DNA Sequences in Laryngeal SCC

The individual specimens were amplified by PCR using pU-1M/pU-2R primer pair for high-risk HPVs and pU-31B/pU-2R primer pair for low-risk HPVs, separately. HPV DNA sequences were detected in 60 (58.8%) of 102 cases (Fig. 1 and Table II). The high-risk HPV-16, -18, and -33 DNAs were detected in 30 cases, 22 cases, and one case (29.4%, 21.6%, and 0.98% overall; 50%, 36.7%, and 1.67% of HPV-positive cases), respectively. The low-risk HPV-6 and -11 DNAs were detected in 25 cases and two cases (24.5 and 1.96% overall; 41.7% and 3.3% of HPV-positive cases), respectively. Both HPV-6 and -16 DNAs were detected in eight cases (7.84% overall; 13.3% of HPV-positive cases) and both HPV-6 and -18 DNAs were detected in 12 cases (11.8% overall; 20% of HPV-positive cases). Thus, coinfection with low- and high-risk HPVs were observed in 20 cases (19.6% overall; 33.3% of HPV-positive cases). In 42 cases, no HPV DNA was detected. The detection of HPV DNA sequences was not significantly correlated with sex, age, tumor site, metastasis to cervical lymph node, and histological type (data not shown).

Detection of p53 Protein in Laryngeal SCC

Overall, 60 (58.8%) of the 102 cases had nuclear p53 over-expression in at least 1% of tumor cells (Fig. 3). Of the p53-positive 60 cases, 26 (43.3%) had nuclear staining in less than 20% of the tumor cells, eight (13.3%) had staining in more than 20% of the tumor cells, and 26 (43.3%) had staining in more than 70% of the tumor cells. No nuclear staining was detected in 42 cases.

There was no significant difference in p53 over-expression between males and females, and among the tumor sites. p53 over-expression was detected in seven cases (87.5%) of eight poorly-differentiated SCC, 43 cases (63.2%) of 68 moderately-differentiated SCC, and 10 cases (41.7%) of 24 well-differentiated SCC (Fig. 2). Thus, p53 was significantly detected in poorly-differentiated SCC ($P < 0.05$). In addition, 30 cases (78.9%) of the 38 SCC with metastasis to cervical lymph nodes were positive for p53 over-expression ($P < 0.05$).

Relationship Between HPV Detection and p53 Over-Expression

Both HPV and p53 were positive in 35 cases (34.5%) of 102 patients, and negative in 17 patients (16.7%; Fig. 4). Either HPV or p53 was positive in 50 cases (25 cases each). Although p53 was detected in 35 (58.3%) of 60

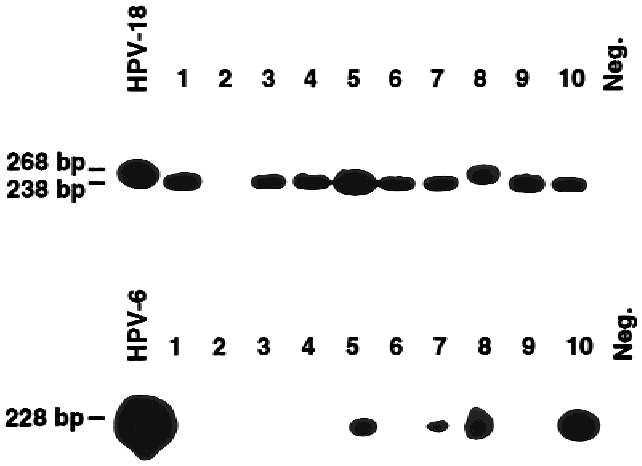

Fig. 1. Detection of HPV DNA sequences in laryngeal SCC. The individual specimens were amplified by PCR using two primer pairs as described in the text. After electrophoresis on 2% agarose gel, HPV DNA sequences were detected by Southern blot hybridization. The cloned HPV-6 and -18 were used as positive controls, respectively. Cases 1, 3, 4, 6, and 9 were positive for HPV-16, but case 2 was negative for HPV. Cases 5, 7, and 10 were positive for both HPV-16 and HPV-6. Case 8 was positive for both HPV-18 and HPV-6.

TABLE II. Typing of HPV in Laryngeal Carcinoma in Northeast China

Type	Cases (% of positive case)
6	5 (8.3)
11	2 (3.3)
16	22 (36.7)
18	10 (16.7)
33	1 (1.7)
6 + 16	8 (13.3)
6 + 18	12 (20.0)
Total	60 (100)

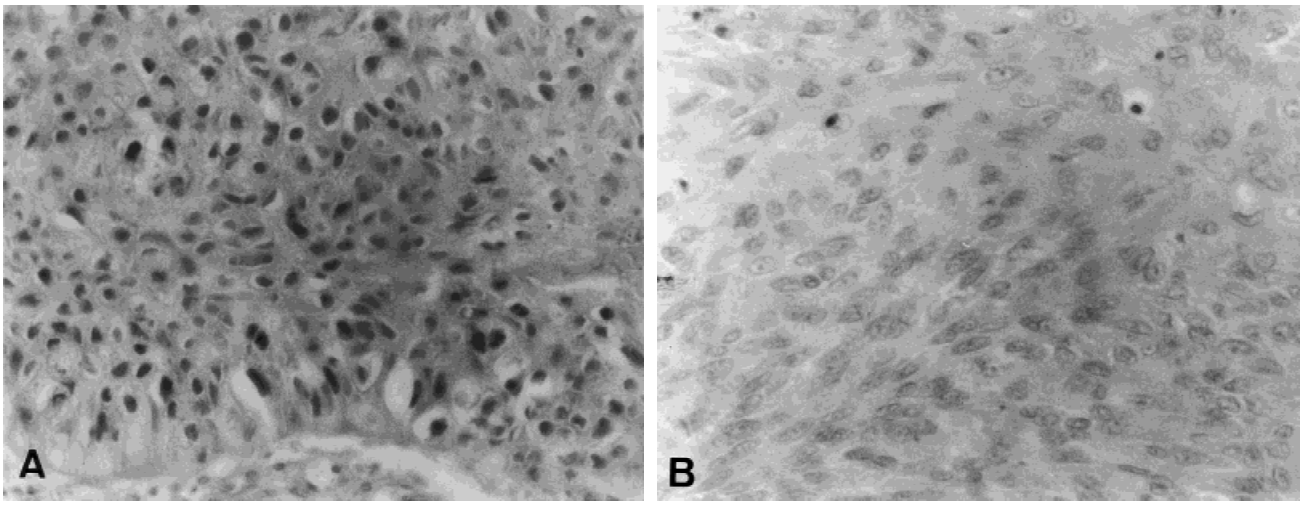

Fig. 2. Immunohistochemical staining of laryngeal SCC. Section was stained either with monoclonal antibody D07 against p53, showing intense nuclear staining of the tumor cells **(A),** or with normal mouse serum **(B)** of p53-positive case. × 200.

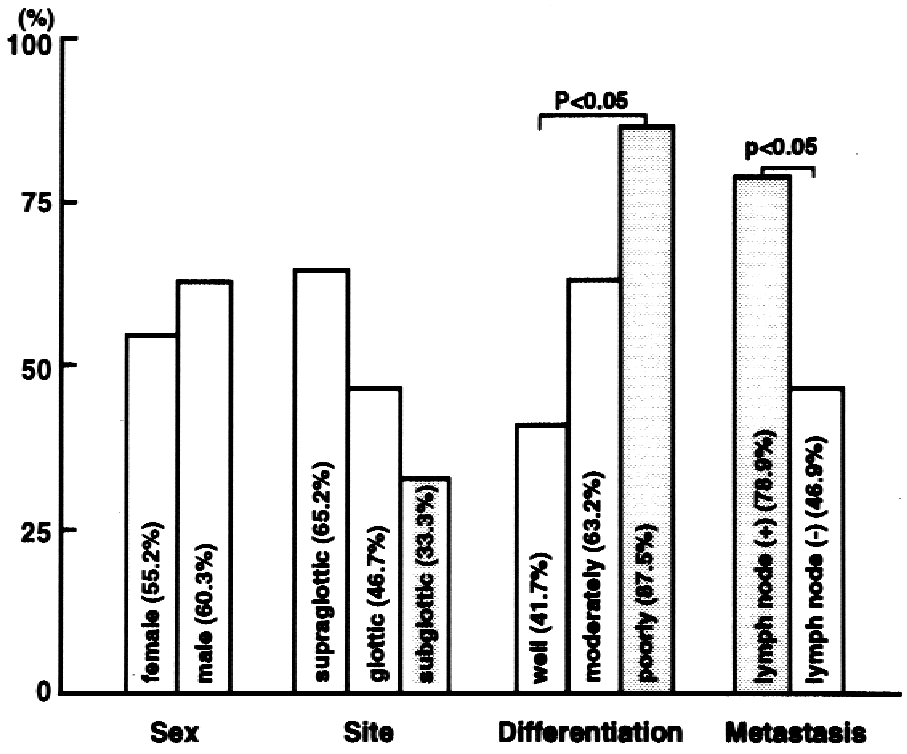

Fig. 3. Characteristics of laryngeal SCC according to p53 over-expression. p53 was significantly over-expressed in poorly-differentiated SCC ($P < 0.05$) and in cases with metastasis ($P < 0.05$), respectively. Statistical significance was analyzed via chi-square test.

HPV-positive cases, there was no significant correlation between HPV detection and p53 over-expression in laryngeal SCC of Northeast China.

DISCUSSION

This large-scale study showed that high-risk HPV-16, -18, and -33 DNAs were positive in 51% of 102 laryngeal SCC in Northeast China. This was almost comparable to that of Perez-Ayala et al. [1990], who detected HPV-16 DNA in 54.2% of 48 Spanish laryngeal SCC. However, the prevalence of HPV infection in laryngeal SCC varied with individual investigations [Kiyabu et al., 1989; Hoshikawa et al., 1990; Anwar et al., 1993; Gorgoulis et al., 1994]. Recently, Brandwein et al. [1993] and Lie et al. [1996] reported that only 8% of laryngeal SCC in USA and in Norway contained HPV DNA, respectively. They suggested that the high detection ratio of HPV in laryngeal SCC resulted from con-

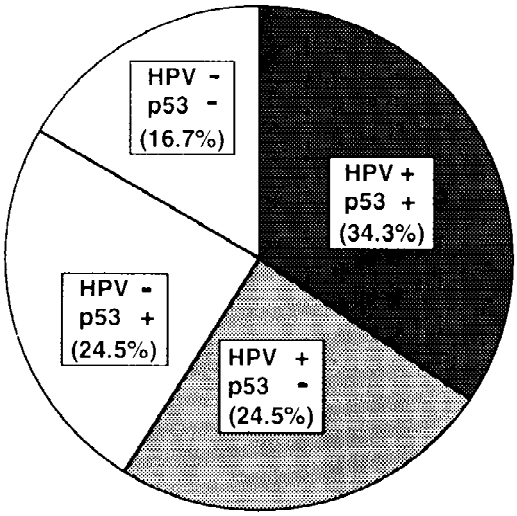

Fig. 4. Relationship between HPV infection and p53 over-expression. Although 35 (58.3%) of 60 HPV-positive cases were also positive for p53 expression, there was no significant correlation between HPV infection and p53 over-expression.

tamination. In this study, however, every effort was made to avoid contamination to the same degree as they did. In addition, we repeated the same experiment, including the preparation of DNA, which showed the same results. Therefore, the difference in the prevalence of HPV infection may partly depends on the primers used in individual studies. Alternatively, personal habit may affect the prevalence of HPV infection. Indeed, HPV infection was observed in 74% of oral cancers of Indian betel quid chewers [Balaram et al., 1995]. In Northeast China, almost 80% of patients with laryngeal SCC were smokers, and 54% had an average Brinkman index of over 300 (data not shown).

Since Scheurlen et al. [1986] detected HPV-16 DNA in laryngeal SCC, HPV-16 has been the most frequently detected in this disease in various geographical regions. On the other hand, HPV-18 DNA has been detected in a few laryngeal SCC cases. [Watts et al., 1991; Simon et al., 1994; Gorgoulis et al., 1994]. Anwar et al. [1993], however, detected HPV-18 and -16 DNAs in 87.5% and 18.8% of Japanese HPV-positive laryngeal SCC, respectively. Although HPV-16 was detected in 50% of HPV-positive cases in Northeast China, HPV-18 DNA was also detected in 36.7% of them. Therefore, HPV-18 was also more prevalent in Northeast China than in other geographical regions, except for Japan.

HPV-6 was usually detected in carcinoma ex-papilloma and in several laryngeal SCC. In Northeast China, HPV-6 infection was detected in 25 cases (41.7% of HPV-positive cases). Five cases were infected with HPV-6 alone and the other 20 cases were coinfected either with HPV-6 and -16 or with HPV-6 and -18. The different biological activities between high-risk and low-risk HPVs were found to be mainly due to the different binding affinity of the E6 and E7 proteins to the tumor suppressor proteins p53 and pRb, respectively [Dyson et al., 1989; Heck et al., 1992]. Recently, Grassmann et al. [1996] found that E6 and E7 proteins of HPV-6 from carcinomas had amino acid exchanges unique for isolates from carcinomas. Although the biological significance of these mutations were still obscure, it is of interest to investigate whether HPV-6 from laryngeal SCC of Northeast China has those amino acid exchanges in E6 and E7 proteins.

p53 over-expression in laryngeal SCC of Northeast China was comparable to those of Japanese and Greek laryngeal SCC [Anwar et al., 1993; Gorgoulis et al., 1994]. In both Northeast China and Japan, p53 was over-expressed significantly in poorly-differentiated SCC and in cases with metastasis, while Gorgoulis et al. [1994] could not find such a significance between differentiation grade of tumor and p53 expression. It has been reported that p53 and pRb were of the wild-type in HPV-positive cervical cancer lines, while both were mutated in two HPV-negative cervical cancer cell lines [Crook et al., 1991]. Similar results were obtained for HPV and p53 in cervical carcinoma tissues [Crook et al, 1992]. However, p53 over-expression was detected recently in 59% of HPV-positive cervical carcinomas [Borreson et al., 1992] and in 69% of HPV-positive laryngeal SCC [Anwar et al., 1993]. p53 over-expression was also detected in 58.3% of HPV-positive laryngeal SCC. These results are not in accordance with the fact that wild-type p53 can be degraded by forming a complex with E6 oncoprotein through ubiquitin-mediated proteolysis [Scheffner et al., 1990]. Because p53 gene expression was investigated only by immunohistochemical staining, the further analysis of p53 gene in HPV-positive and -negative laryngeal SCC is required for the evaluation of whether mutation(s) of p53 gene really exists in HPV-positive cases.

In conclusion, although HPV DNA and p53 were detected in 58.8% of laryngeal SCC, respectively, there was no significant correlation between HPV infection and p53 over-expression. Further studies such as environmental, hormonal, and genetic analyses are required for understanding the genesis in laryngeal SCC of Northeast China.

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